## Supporting Information

# Autonomous Application of Quantitative PCR in the Deep Sea: In situ Surveys of Aerobic Methanotrophs using the Deep-sea Environmental Sample Processor

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MATERIALS AND METHODS

**Image of seafloor at the MARS deployment site**

Figure S1-S2. An ROV-acquired image illustrates the characteristic features of the seafloor at the MARS D-ESP deployment site. The seafloor at the MARS site comprises relatively smooth, but firm, tan hemiplegic sediment with a biologically diverse and relatively abundant benthic fauna. Dominate sessile organisms include the sea pens *Funiculina sp.* and *Umbellula lindali,* along with mobile fishes, skates, crabs, and gastropods. Width of the skate is approximately 1 meter.

**Development of qPCR 5'-nuclease assays for the *in situ* D-ESP deployment**

Target sequences for OPU1 and OPU3 *pmo*A and 16S rRNA qPCR assay development (Table S1-S4) were recovered from particulate samples filtered from seawater samples collected by CTD-rosette casts and ROV Niskin bottles at and nearby the Santa Monica mound in 2005 (Tavormina et al. 2008). Quantitative PCR assays were developed initially at the California Institute of Technology using an Applied Biosystems 7500 real-time PCR system using a two-

step PCR (94 °C, 15 s and 60 °C, 60 sec) for 40 cycles, and the TaqMan® Universal PCR Master Mix from Applied Biosystems. Standard curve analysis using CsCl-purified linearized plasmid templates over 5 orders of magnitude indicated amplification efficiencies >95% for all targets. For the *in situ* D-ESP deployment qPCR 5'-nuclease assays for OPU1 and OPU3 *pmoA* were conducted according to Tavormina et al. (2010), and the 16S ribosomal assays were conducted according to Tavormina et al. (2013). Tests of the qPCR assays and generation of standard curves for the *in situ* deployment used slightly different primer/probe concentrations. Final concentrations of primer and probe sequences for each phase of development are listed below in the qPCR methods notes.

**Nucleic acid extraction protocol after Varaljay (2012)**

Frozen filters were thawed briefly and transferred with biomass side facing inward to 2 mL screw cap tubes with an integral o-ring containing 250 μL of cleaned zirconium beads (equal volumes of bleach-cleaned then ashed per manufacturer's instruction, 0.5 and 0.2-μm diameter; BioSpec Products, Bartlesville, OK). 750 mL RLT Plus lysis buffer (Qiagen, Valencia, CA) with β-mercaptoethanol (mixed daily; Fisher Scientific) was added to each tube. For filters stabilized with RNA*later*® (Life Technologies, Carslbad, CA) NaOH was added to the lysis buffer (10 μL 1N NaOH per 1 mL of RLT Plus lysis buffer w/β-ME) to adjust the pH. Tubes containing the filter, beads, and lysis buffer were beaten for 2 minutes using a BioSpec Model MBB-8 bead beater at maximum speed. Tubes were centrifuged at 13,000 rpm for 1 minute to pellet the beads. Lysate was filtered through a 13-mm diameter 0.2 μm PVDF syringe filter (Millex GV, Millipore Corporation, Billerica, MA) using a 10-mL disposable syringe, and a 500 μL aliquot was used as the initial starting material for the Qiagen AllPrep DNA/RNA Mini Kit. The

remainder of the protocol was per manufacturer's instructions except that RNA and DNA from the respective column were eluted in 50 μL RNAse-free water or 60 μL EB buffer (Qiagen), respectively, and stored at -80°C until use.

Table S1-S5. Primer and probe sequences used for qPCR 5'-nuclease assays.

| | OPU1 *pmo*A gene | OPU3 *pmo*A gene | OPU1 16S rRNA gene | OPU3 16S rRNA gene |
|---|---|---|---|---|
| Forward primer [a] | pmoA_OPU1qPCR_242f TTACCCCGATCATGCTGGTT | pmoA_OPU3qPCR_492f TTGCACCTTTACATYTACCTGTTGA | 16S_OPU1qPCR_f CAATGCCGCGTGTGTGAA | 16S_OPU3qPCR_f AGCACTTTCAATTGGGAGGAAA |
| Reverse primer [a] | pmoA_OPU1qPCR_312r GATTCTGAAGTGTTCCCAAACGA | pmoA_OPU3qPCR_612r ACCTTTTTCTACCATTCYGATRTACTC | 16S_OPU1qPCR_r CCTCTCTTCCTCCCGACTGAA | 16S_OPU3qPCR_r GCCGGTGCTTCTTCTAAAGGT |
| 5'-nuclease probe [a] | pmoA_OPU1_probe TTCCCAGCCGCTGTTCAGGCA | pmoA_OPU3_probe ACAACGGCATGATGTTTACTGTTGCTGATTTA | 16S_OPU1_probe AGGCCTGCGGGTTGTAAAGCA | 16S_OPU3_probe MAGCTGGGTTAATAGCCCYGCTCTTGACA |
| Assay reference | Tavormina et al. (2010) | Tavormina et al. (2010) | Tavormina et al. (2013) | Tavormina et al. (2013) |

[a] primer and probe concentrations used for benchtop qPCR and *in situ* D-ESP assays varied (see below).

**Notes for qPCR methods**

*Benchtop Assay Development*

Optimum results were obtained at the following final concentrations in a 30 μL reaction volume for all four target genes: 1 μM forward primer, 1.5 μM reverse primer and 200 nM of the 5'-nuclease assay probe labeled with 6-carboxyfluorescein (FAM, 5') and a black hole quencher (BHQ, 3'). Primers (Integrated DNA Technologies, Coralville, IA) and probes (Sigma Aldrich, St. Louis MO) were resuspended in TE buffer (10mM Tris and 1 mM EDTA; pH 8.0) for long-term storage in individual-use aliquots at -80°C.

*D-ESP in situ*

Optimum results for *in situ* analysis using the qPCR module were obtained under slightly different conditions than for the benchtop assays: 1 μM (OPU1 *pmo*A, OPU3 *pmo*A and OPU1 16S rRNA) or 1.3 μM (OPU3 16S rRNA) forward primer, 1.3 μM (OPU1 *pmo*A) or 1 μM (OPU3 *pmo*A, OPU1 16S rRNA, and OPU3 16S rRNA) reverse primer, and 100 □M (OPU1 *pmo*A) or 130 □M (OPU3 *pmo*A, OPU1 16S rRNA, and OPU3 16S rRNA) of the 5'-nuclease probes labeled with 6-carboxyfluorescein (FAM, 5') and a black hole quencher (BHQ, 3') (Operon Biotechnologies, Huntsville, AL). Two additional qPCR assays included an internal positive control (IPC) as described by Preston et al. (2011) and the Group 1 marine archaea (G1Ar; data not shown). TE buffer was used to dilute primer/probe solutions to 5x final concentration prior to loading in the reagent coils. Enzyme mix 1 [2x AccuPrime™ SuperMix I (Life Technologies, Valencia, CA) with a final concentration of 4.0 mM $MgCl_2$] was utilized for the IPC and G1Ar assays, and enzyme mix 2 (2x AccuPrime™ SuperMix I with a final concentration of 5.5 mM $MgCl_2$) was used for the four OPU qPCR assays. Primer/probe and enzyme mixes were stored separately in 20% bleached-cleaned reagent coils to extend useful

shelf-life of the reagents. Previous experience has shown that qPCR reagents stored in this configuration are stable at room temperature for up to 10 months.

*Post-Deployment Assays*

Verification of qPCR results obtained *in situ* and RT-qPCR assays of paired DNA and RNA fractions extracted from the same preserved filter were run in parallel on the same sets of 96-well plates. Final primer/probe concentrations were the same as listed above for the D-ESP *in situ* assays, however the primers and probes were from a different manufacturer. Primers and probes (Integrated DNA Technologies, Coralville, IA) were resuspended in molecular biology grade water (Sigma, St Louis, MO) before use. The enzyme mix was prepared by adding a magnesium chloride solution (Sigma, St. Louis, MO) to AccuPrime™ SuperMix I enzyme (Life Technologies, Valencia, CA) to obtain a final concentration of 5.5 mM. To ensure that internally consistent results were obtained for each target assay, a single mixture of primers, probe, and enzyme mix was prepared prior to qPCR analysis of both the DNA extracts and cDNA. Prior to cDNA synthesis, bench top surfaces and pipetters were pre-cleaned with RNaseZap® (Life Technologies, Carlsbad, CA) and rinsed with molecular biology grade water.

Two 96-well plates were required for each target gene assay and a full suite of standards and a no template control were included on each plate. Quantitative PCR assays were obtained on an Applied Biosystems StepOnePlus™ instrument using a two-step reaction comprising an initial hold at 95°C for 2 min, followed 40 amplification cycles of denaturation at 95°C for 15 sec and hybridization and extension at 60°C for 1 min. DNA contamination of the cDNA products was not detected in any of the RT-qPCR NRT assays. No template control (NTC) assays using DEPC-treated water (Life Technologies, Carlsbad, CA) did not detect target DNA or cDNA. Similarly, RT-qPCR assays of a field blank filter archived by the D-ESP (obtained five days

prior to deployment of the D-ESP on the Santa Monica mound by filtering 0.5 L molecular biology grade water) or a lab blank filter (25-mm-diameter 0.22 μm Durapore processed in parallel with archived filters from the D-ESP) did not detect any target DNA or cDNA.

Table S2-S8. Summary of qPCR standard curve parameters for the D-ESP *in situ* and post-deployment with an ABI StepOnePlus™ instrument.

*In situ* gene abundance assays

| Gene target | Standard Concentrations (targets/mL)[a] | Slope | Intercept | Linear Regression Coefficient ($r^2$) | Amplification efficiency[a] |
|---|---|---|---|---|---|
| OPU1 16S rRNA | $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$ | -3.48 | 38.993 | 0.997 | 94.7 |
| OPU1 *pmo*A | $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$ | -3.43 | 39.029 | 0.995 | 95.8 |
| OPU3 16S rRNA | $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$ | -3.43 | 39.312 | 0.994 | 95.8 |
| OPU3 *pmo*A | $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$ | -3.44 | 41.277 | 0.996 | 95.3 |

Post-deployment StepOnePlus™ assays

| Gene target | Standard Concentrations (targets/mL) [b] | Slope | Intercept | Linear Regression Coefficient ($r^2$) | Amplification efficiency[a] |
|---|---|---|---|---|---|
| OPU1 16S rRNA plate 1 | $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$ | -3.4225 | 36.103 | 0.999 | 96.0 |
| OPU1 16S rRNA plate 2 | $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$ | -3.3830 | 36.108 | 0.999 | 97.5 |
| OPU1 *pmo*A | $1x10^1$, $1x10^2$, $1x10^3$, | -3.3947 | 36.727 | 0.999 | 97.0 |

| | | | | | |
|---|---|---|---|---|---|
| plate 1 | $1x10^4$, $1x10^5$, $1x10^6$ | | | | |
| OPU1 *pmo*A plate 2 | $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$ | -3.3351 | 36.480 | 0.999 | 99.4 |
| OPU3 16S rRNA plate 1 | $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$ | -3.4112 | 37.979 | 0.999 | 96.2 |
| OPU3 16S rRNA plate 2 | $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$ | -3.3606 | 37.791 | 0.999 | 98.4 |
| OPU3 *pmo*A plate 1 | $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$ | -3.4804 | 37.283 | 0.999 | 93.8 |
| OPU3 *pmo*A plate 2 | $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$ | -3.4618 | 37.228 | 0.999 | 94.5 |

[a]efficiency = $10^{(-1/slope)} - 1$

[b]The same stock standard was used for the *in situ* deployments and post-deployment gene expression assays.

**ISMS operation and calibration**

The ISMS was assembled and configured as described in Wankel et al. (2011), except for slight modifications necessary for integration into the fluid sampling flow path (Figure 4). A Seabird 5T pump was used draw seawater across the membrane inlet of the ISMS and through other downstream contextual sensors. Dye tracer experiments indicated that water flowed from the system inlet to pump outlet within 15 sec. Mass spectrometer measurements occurred every 10 minutes for a 1-minute duration. This measurement was initiated 30 seconds after activating the pump to ensure that the sample line was completely flushed.

Concentrations of chemical species detected using the ISMS are based on 8-minute integrations that captured the rise and decay of methane concentrations within the pumped sampling interval. Methane was measured at m/z 15, and normalized to water at m/z 18 and dissolved nitrogen at m/z 28 to remove baseline drift that occurs during short-term deployments.

Laboratory calibrations of the ISMS were performed using high-pressure pumps (Beckman-Coulter, Fullerton, CA) that delivered seawater with known dissolved methane concentrations past the membrane inlet. Calibration fluid and membrane inlet temperatures were controlled by immersion in a temperature-controlled waterbath (±0.1˚C). Hydraulic pressure was controlled by a backpressure regulator (StraVal Valve, Garfield, NJ). Multiple laboratory calibrations have shown that Teflon™ AF membrane inlets are resistant to change under large ranges of hydrostatic pressure. Measurement accuracy of methane concentration is approximately 10%, while the analytical precision is less than 1%.

**Water column methane sample collection and analysis**

Water samples were collected using either a CTD-rosette with 10-L Niskin bottles, or ROV-mounted 5-L Niskin bottles. Immediately after return to the surface, the water was transferred via Tygon tubing to 250-mL bottles and sealed with an open-top screw cap and septum (polytetrafluoroethylene-silicone septum; Supelco, Bellefonte, PA) for shipboard gas chromatographic analysis. Methane gas for chromatograph analysis was extracted from the bottle samples by headspace equilibration. The water sample was thermally equilibrated to laboratory temperature by positioning the bottle on its side, and inserting a 22-gauge needle into the septum to relieve pressure created during thermal expansion. After equilibration, the bottle was inverted and a 10-mL aliquot of ultra-high purity (UHP) nitrogen gas was injected into the bottle, expelling an equivalent amount of water through the thermal equilibration needle. The bottles were shaken inverted for 5 minutes to equilibrate the headspace with the water using an air-operated paint mixer. Bottles were then oriented upright, and the headspace water transferred to a 10mL syringe by fluid displacement. Methane gas concentration in the syringe samples was

determined using a Shimadzu mini-2 gas chromatograph equipped with a flame ionization detector. A small volume magnesium perchlorate drying trap removed water vapor prior to introduction into the sample injection valve. Gases were separated using a 5 ft x 1/8" OD stainless steel Carbosieve G packed column (80-100 mesh; Supelco, Bellefonte, PA) and an UHP nitrogen carrier gas. Oven and detector temperatures were 100°C and 125°C respectively. Methane gas standards (9.93 and 98.6 ppm in nitrogen; Scott Specialty Gases, Plumsteadville PA) were used for calibration. Standard error of analysis was generally less than 1% for standards and replicate samples. Replicates analyses were performed on separate subsamples of the Niskin bottle. The methane detection limit using this method was ~0.2 nM.

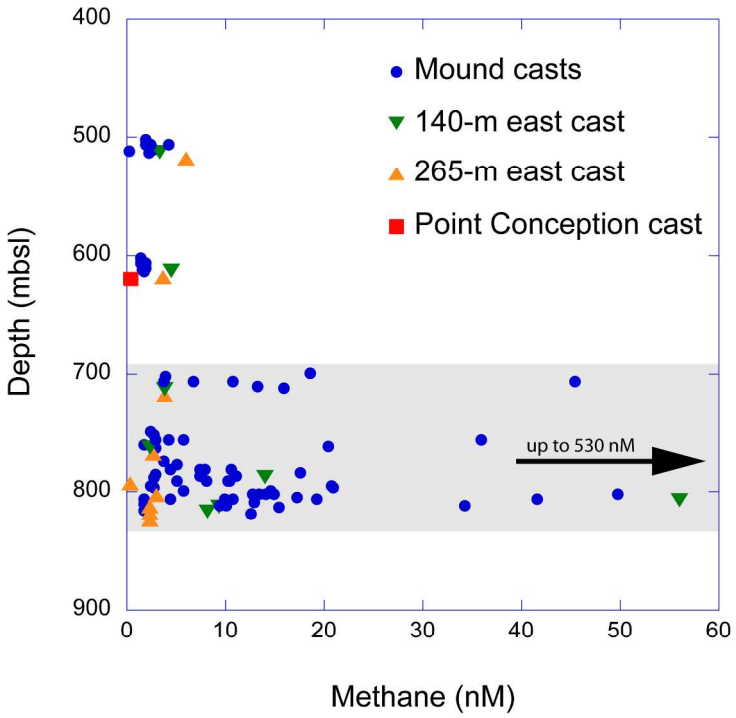

Figure S2-S11. Methane concentrations for CTD-rosette-collected water column cast samples from above and surrounding the Santa Monica mound and near the two off-mound D-ESP deployment sites show a high degree of spatial variability and substantial enrichment above background ocean values (<1 nM) in a depth zone between 690 meters below sea level (mbsl) and the seafloor (highlighted by gray shading). Methane concentrations up to 530 nM were measured in the water column immediately adjacent to the crest.

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